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Award Number: DAMD17-01-1-0564

TITLE: Identification of Novel Mitotic Checkpoint Genes in Breast Cancer

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REPORT DATE: May 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2002	Final (1 May 01 - 30 Apr 02)

4. TITLE AND SUBTITLE
Identification of Novel Mitotic Checkpoint Genes in Breast Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0564

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Malignant progression in mammary epithelial cells stems from the accumulation of multiple mutations in tumor suppressor genes and protooncogenes. It is widely hypothesized that these mutations arise in part due to some degree of genomic instability. Given most breast cancer cells demonstrate some degree of aneuploidy suggesting chromosomal instability, we wanted to examine breast cancer cells for the relationship of chromosomal instability to alterations in genes and proteins that regulate the mitotic spindle checkpoint. We first characterized the degree of chromosomal instability in breast cancer cells and examined the cells for alterations in known spindle checkpoint genes (*MAD2L1*, *MAD1L1*, *BUB1*, *BUBR1*, and *CDC20*) and their related proteins. We then searched for novel proteins that interact with spindle checkpoint proteins and identified *EB1*. While few alterations were found in *BUB1*, *BUBR1*, *EB1*, and *CDC20*, we found decreased transcript and protein expression of *MAD1L1* and *MAD2L1* in breast cancer cells. Mutation analysis of these genes reveals some alterations that may drive malignant progression. Further *in vitro* and *in vivo* analyses are underway to characterize how alterations in spindle checkpoint genes may contribute to mammary carcinogenesis.

14. SUBJECT TERMS
Breast Cancer, Mitotic checkpoint, *MAD2*, chromosome instability, spindle checkpoint, *BUB*, mitotic arrest

15. NUMBER OF PAGES
14

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

While mutations in proto-oncogenes and tumor suppressor genes have been identified in breast cancer cells, the molecular etiology of breast cancer remains poorly understood. In somatic mammary epithelial cells lacking predisposing germline mutations, it is hypothesized that the occurrence of sufficient mutations to cause malignant transformation is improbable without genomic instability (1-10). Most breast cancers are aneuploid, suggesting that some type of chromosome instability likely accelerates tumorigenesis (11-25). Aneuploidy can result from mitotic spindle checkpoint alterations (26-43). When normal cells divide, chromosomes faithfully segregate after chromosome attachment to mitotic spindle microtubules. Genetic events disrupting the mitotic spindle checkpoint may allow cells to proliferate with misaligned chromosomes resulting in chromosome instability and aneuploidy. Mitotic spindle checkpoint proteins, BUB (budding uninhibited by benomyl) and MAD (mitotic arrest-deficient) proteins, have been described in human cancers (44-49) . For example, aneuploid colon cancer cells with *BUB1* and *BUB1B* mutations do not arrest in mitosis when microtubules are chemically disrupted (26,44). Furthermore, transfection of mutant *BUB1* in euploid colon cells disrupts the checkpoint and causes aneuploidy. Low *MAD2L1* expression was previously noted in a breast cancer line with a defective mitotic checkpoint (50). The active form of the *MAD2L1* protein (MAD2) localizes within a BUB/MAD complex to unattached chromosomes (51-57). When chromosomes are incorrectly aligned, MAD2 helps activate a mitotic checkpoint. It is thought that upon activation, the carboxy end of MAD2 sequesters CDC20 thereby inhibiting the anaphase promoting complex, preventing anaphase (58-61). When chromosomes are properly aligned, the MAD/BUB complex dissociates, MAD2 releases CDC20 and cells proceed through anaphase. As predicted, microinjection of MAD2 Ab causes premature anaphase while excess MAD2 causes arrest (62). *MAD2L1* maps to 4q27 where over 50% loss of heterozygosity (LOH) in breast and other cancers has been demonstrated (63-67). *BUB1B* maps to 15q14, which also displays LOH in breast cancer (68). Interestingly, in Brca2 deficient murine cells, mutant Bub1, Bub1B (Mad2L3), and p53, potentiate cellular growth and transformation suggesting that inactivating checkpoint gene mutations cause transformation (69). *BRCA2* associates with *BUB1B in vitro* only in cells with disrupted mitotic spindles (70). We hypothesized that other putative spindle checkpoint genes could be important in breast cancer. Specifically, we hypothesized that altered expression of MAD2 and/or other mitotic spindle checkpoint proteins may allow anaphase progression with chromosome attachment errors that would drive malignant progression in mammary epithelial cells. The goal of our Concept Proposal was to further characterize the mitotic spindle checkpoint in breast cancer and begin to identify genes involved in the process.

Body:

The overall goal of stated in our Concept Proposal was to "isolate and characterize those spindle checkpoint genes involved in breast cancer and begin to determine how alterations of these genes may contribute to mammary tumor progression by in vitro analysis of well-characterized human breast cancer samples." While the format of the Concept Award did not include a formal Statement of Work, we proposed to do this by determining what functional gene alterations occur in breast cancer cells associated with aneuploidy and chromosome instability. Before fishing for involvement of novel genes, we first wanted to identify the degree of aneuploidy and chromosomal instability in a well-characterized panel of breast samples and analyze them for involvement of known mitotic spindle checkpoint gene alterations. Our initial analysis of 100 primary breast cancers and matched normal tissue with 10 microsatellite markers confirmed that microsatellite instability is rare in primary breast cancers (< 3%) and supporting our hypothesis that other types of genetic instability likely contribute to malignant progression in breast cancer cells.

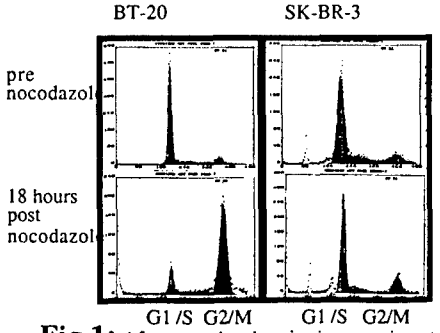


Fig 1: After nocodazole mitotic arrest is noted in BT-20. not SK-BR-3 cells

We examined the mitotic checkpoint in 20 ATCC and SUM breast cancer and 5 immortalized normal human mammary epithelial cell lines. Early passage SUM breast cancer lines and HPV immortalized normal mammary epithelial lines had been previously characterized for comparative genomic hybridization patterns, hormone receptor status, growth factor dependence, and some genetic alterations (71-73). The breast cancer lines, immortalized mammary lines, and four control colon cancer lines (2 euploid with normal checkpoints, 2 aneuploid with abnormal checkpoints) were treated with nocodazole and paclitaxel (Taxol),

microtubule disrupting agents causing mitotic arrest in normal cells via different mechanisms. Cells pre- and post-nocodazole, collected q 6 hrs x 36 hrs, were assessed by flow cytometry for cell cycle distribution, mitotic index, and ploidy. In euploid controls, normal checkpoints caused peak miotic arrest with condensed chromosomes at approximately18 hrs after treatment. Cells with presumed abnormal checkpoints did not arrest (see example in fig 1). Three fourths (15/20) of aneuploid breast cancer lines lacked a normal checkpoint. All immortalized lines, except MCF10, arrested normally. The few highly aneuploid lines that did not arrest suggest possible drug resistance or alternative instability mechanisms.

Since *BUB1* and *BUB1B* mutations were found in colon cancer cells and demonstrated intriguing *BRCA2* associations, prior to the start of this proposal we initially screened breast cancer cells for alterations of these genes by Northern blot and mutation screening analyses of cDNA. No significant alterations were identified (68). This suggested that *BUB1* and *BUB1B* alterations are infrequent in breast cancer, and led us to look more carefully at MAD2 and MAD2-related proteins during the course of this proposal. The specific genes and proteins we analyzed in detail during the course of this proposal are listed in the following table.

Gene ID	Gene name	Location	Characteristics	Protein ID	Size	General function at the MSC
MAD1L1	mitotic arrest deficient 1, S. cerevisiae, homolog-like 1	7p22	16 exons spans 1990 bp 1983 ORF	**MAD1 or TBXP181**	803 aa	Helps prevent anaphase until all chromosomes are properly aligned at the metaphase plate. Brings MAD2 into MSC complex. Putative target for type 1 T cell leukemia virus oncoprotein Tax
MAD2L2	mitotic arrest deficient 2, S. cerevisiae, homolog-like 1	4q27	5 exons spans 1382 bp 693 ORF	**MAD2 or MAD2A**	205 aa 25 kDa	Regulates onset of anaphase via monitoring kinetochore/spindle attachments at metaphase plate. Sequesters CDC20, thereby preventing anaphase.
CDC20	cell division cycle 20, S. cerevisiae homolog	9q12-22	11 exons spans 1680 bp 1500 bp ORF	**CDC20 or p55CDC**	499 aa 55kD	Activates the anaphase promoting complex by activating cyclin ubiquitination directed degradation of D-box containing substrates
MAPRE1	microtubule associated protein, RP/EB family, member 1	20q11.1-q11.23	7 exons spans 2546 bp 870 bp ORF	**EB1**	290 aa 30-35 kDa	Part of microtubule cytoskeleton and centrosomes during interphase; localized to end of mitotic spindle, binds to APC. Complexes with MAD2.

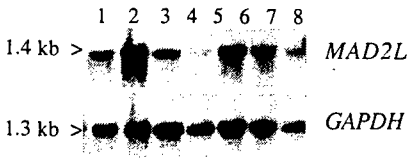


FIG 2: Nothern blot: decreased *MAD2L1* expression in HS573T (4), increased expression in CAL51 (2)

MAD2L1 alterations were identified in breast cancer cells. MDA-MB-435s, Hs578T, MDA-MB-231, BT-474, and DU4475 exhibited < 50% expression of *MAD2L1* as confirmed by densitometry analysis (Digital Imaging System, Alpha Innotech) (see example in fig 2). No aberrantly sized transcripts were present. Interestingly, CAL51 (a minimally aneuploid breast cancer line with tetraploidy from J. Gioanni, France) demonstrated 3X higher *MAD2L1* expression compared to immortalized breast epithelial cell lines on Northern blots after standardizing with loading controls. As several experimental factors can cause variable transcript expression the significance of low expression was uncertain. *MAD2L1* cDNAs from these lines were screened for mutations. All sequence variants were confirmed in new cDNA samples. Most were polymorphisms. One unique 3'UTR variant was detected in Hs578T, a line with decreased *MAD2L1* expression, suggesting it may alter transcript stability or processing. Significantly, a heterozygous (het) frameshift mutation, deletion of A at base 572 (Δ572) was noted in CAL51 cells. It is predicted to change 27 carboxy amino acids (aa) and cause loss of 13 terminal aa due to a premature stop codon. Small DNA fragments from old acid-fixed archival normal and primary tumor tissue slides for CAL51 could not be adequately amplified with genomic primers to test primary tissues. CAL51 has a high proportion of tetraploid cells. This het Δ572 *MAD2L1* mutation (MU) causing checkpoint dysfunction identified in breast cancer cells was hypothesized to be functionally significant as the 10 terminal amino acids at the 3'end of MAD2 are important for CDC20 sequestration and oligomerization (see Introduction). We hypothesized that a het Δ572 MU could disturb CDC20 binding and MAD2 tetramerization and disrupt the mitotic checkpoint unless other pathways compensate. The increased *MAD2L1* transcript expression seen in CAL51 could suggest compensatory efforts of overexpression of a wild type (WT) allele.

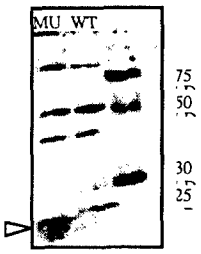


Fig 4 *In vitro* transcription translation assay: shows truncated protein from del 572 A mutant

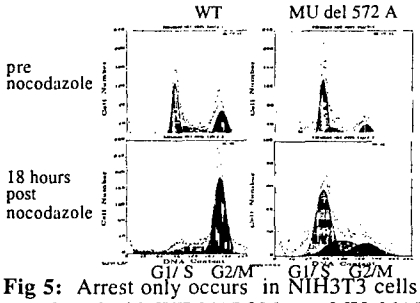


Fig 5: Arrest only occurs in NIH3T3 cells transfected with WT *MAD2L1*, not MU *MAD2L1*

To determine if Δ572 MU generates a truncated MAD2 protein, WT and MU cDNAs were cloned in a pcDNA3.1 expression vector (Invitrogen). *In vitro* transcription/translation (TNT T7 Transcription/Translation System, Promega) revealed a truncated MAD2 protein at 22.2 kD as compared to a 23.5 kD WT protein (fig 4). Δ572 MU, WT, and empty vector constructs were subsequently stably transfected into NIH-3T3 cells (FuGene 6, GibcoBRL). Mitotic checkpoint function and ploidy were examined after nocodazole (see 1-B). WT, vector only, and parental cells demonstrated clear mitotic arrest peaking 18 hours after nocodazole. MU transfected cells did not arrest (fig 6), supporting our hypothesis that het Δ572 has a dominant negative effect on the checkpoint. MU transfectants grew, on average, 2X faster than controls. Assays were done in triplicate with 3 clones of each type.

Western blots of aneuploid cancer lines, were incubated with MAD2 carboxy and amino polyclonal Abs (Santa Cruz) and an β-actin control Ab and detected by chemiluminescence (ECL, Amersham). Two lines displayed significantly diminished expression of the normal 23.5 kD MAD2 protein after controlled for loading with actin. This was confirmed with new proteins from fresh lines. Only lines lacking normal MAD2 expression had abnormal mitotic checkpoints.

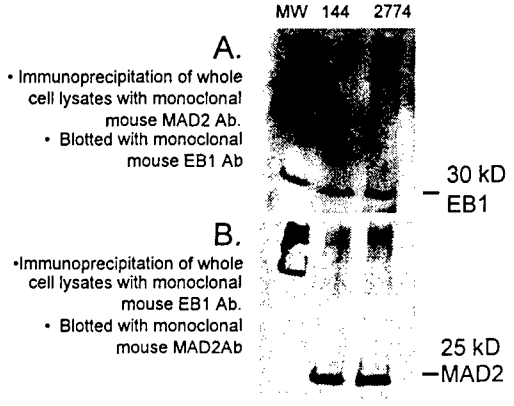
MAD1 protein expression was significantly reduced in numerous breast cancer cell lines. However, there was no clear correlation between lines with low MAD2 expression and checkpoint dysfunction compared to lines with low MAD1 expression suggesting interactions with other proteins. Sequencing of MAD1 exons in breast cancer lines revealed multiple alterations in the conserved coil-coil domain as well as a leucine zipper domain. Some of these sequence alterations suggest pathogenic mutations of functional significance while others are likely new nucleotide polymorphisms. Functional analyses of MAD1 mutations are underway to determine the relationship between loss of MAD1 protein expression and spindle checkpoint dysfunction as well as the relationship between MAD1 and MAD2 in the presence of aberrant MAD1 expression. We hope to assess MAD1 protein expression in primary tumor tissue samples, however, our efforts are limited by the lack of a commercial antibody. Alternate methods will be explored. MAD1 is an important regulator of MAD2. It is hyperphosphorylated in G2/M, which leads to binding and recruitment of MAD2 to the kinetochore when the checkpoint is activated by unattached chromosomes.

To further analyze the role of MAD2 in breast cancer and to develop an in vivo model that we could use to uncover novel spindle checkpoint alterations in breast cancer, we have begun creating a mammary tissue specific transgenic murine model of the *MAD2L1* 572 del A using standard methods (74-77). Recent *in vivo* studies have indicated that Mad2 knockout mice are embryonic lethal at E6.5 and studies carried out on Mad2 knockout mice showed that embryonic cells lacking Mad2 fail to arrest in response to microtubule inhibitors and loss of the checkpoint resulted in chromosome missegregation and apoptosis (78). Subsequently, we have designed a construct with *MAD2L1* 572 del A cDNA, mouse protoamine poly A and intron that is driven by a breast specific promoter, MMTV, to create a transgenic line of mice overexpressing mutant *MAD2L1* 572 del A to examine the effects of this mutation in epithelial breast tissue. Our UM Transgenic Mouse Core injected the construct and 123 mice were initially generated. 23 mice were found to be positive for the transgene by PCR. Southern blot was carried out on all 23 mice. Three male mice were found to carry 5 or greater copies of the transgene while one female appear to be carry one copy of the transgene. The three founder males were subsequently mated to two 6-8 week old BL/6 female. One founder male appears to be unable to sire litter from females after several attempts with different female mice. Female F1 offspring were sacrificed and breast tissue were harvest to test for expression of the transgene. Using a polyclonal MAD2 antibody made from the N-terminus of MAD2 (obtained from Santa Cruz) we are carrying out immunohistochemistry on multiple tissue sections of wildtype and transgenic animals to asses level of MAD2 expression. Mammary tissue from transgenic animals and control litter mates will be compared by expression arrays to help identify novel genes that may be involved in spindle checkpoint regulation. Animals will be followed for tumor formation. Plans to cross transgenic animals with animals having Brca2 and animals with p53 mutations are underway.

Co-immunoprecipitation blots were done to confirm the association between CDC20 and MAD2 (not shown). CDC20 is another component of the mitotic spindle checkpoint pathway that we explored. As noted above, it is required to activate the anaphase- promoting complex thereby initiating anaphase and for the exit from mitosis. *CDC20* transcript expression in breast cancer cells by Northern blot analyses suggest variable transcript expression levels. Sequencing of breast cancer lines demonstrated that five lines shared a common C to A variant in intron 5 (45 bases from splice donor in exon 6) that was not identified in non-cancer cells. It is unclear whether this sequence variant might contribute to an unstable or alternative transcript. Further studies are underway to characterize the role(s) of CDC20 in mammary tumorigenesis.

Given that EB1 localizes to the kinetochore binding ends of spindle microtubules during metaphase and is critically important for targeting the microtubule to the kinetochore (79,80), we hypothesized that MAD2 may interact with EB1 when monitoring unattached kinetochores. MAD2 bead captured immunocomplexes from ovarian cell lysates were subjected to reducing gel electrophoresis followed by immunoblotting using a mouse monoclonal EB1 Ab (BD Transduction laboratories). Similarly, EB1 captured immunocomplexes were electrophoresed and immunoblotted with a mouse monoclonal MAD2 Ab. As shown, MAD2 Ab detected the EB1 immunocomplexes and vice versa, suggesting that MAD2 and EB1 associate in the same protein complex (fig 6). Further analyses of this interaction through GST fusion pull downs and cellular co-localization studies are in progress. Immunohistochemistry staining with nanti-EB1 detects diffuse staining in both nucleus and cytoplasm of most cancer cells and immunoblot analyses shows variable protein expression levels of EB1 in these cells. Given this, we have no further plans to study EB1 in breast cancer cells at present.

Fig 6: Association of MAD2 and EB1 by immunoprecipitation and immunoblotting analyses



Given our above findings we are now focusing our efforts on trying to elucidate what role(s) alterations in MAD2 and MAD2- related spindle checkpoint proteins have in the malignant progression of mammary tumors. During the course of this proposal we have developed in vitro and in vivo models to further characterize the roles of mitotic spindle checkpoint genes in breast cancer checkpoint genes. We are excited out continuing our research efforts in this area of breast cancer research. We are seeking additional funds to continue our work in this area.

Key Research Accomplishments:

During the tenure of this Concept Award, we have:

- Carefully characterized a panel of breast cancer cell lines for ploidy status, spindle checkpoint dysfunction in response to chemicals that disrupt the spindle microtubules, and chromosomal instability.
- Completed detailed transcript expression, protein expression, and mutational analyses of five recognized mitotic spindle checkpoint genes in breast cancer cells demonstrating spindle checkpoint dysfunction and have found alterations in *MAD2L1* and *MAD1L1* that have probable functional significance in the malignant progression of breast cancer
- Conducted functional studies of *MAD2L1* mutations in an in vitro model system and demonstrated that dominant negative alterations of *MAD2L1* identified in a breast cancer cell line can disrupt the mitotic spindle checkpoint.
- Begun in vitro functional analyses of other mitotic spindle checkpoint gene alterations.
- Developed a mammary tissue specific transgenic mouse model of the putative dominant negative *MAD2L1* mutation identified in a breast cancer cell line to further characterize the impact of MAD2 alterations in an in vivo system and to help identify additional spindle checkpoint gene alterations involved in mammary tumorigenesis. In addition, this model should be useful to elucidate how spindle checkpoint alterations interact with mutations in *BRCA1*, *BRCA2*, and *TP53* gene alterations in mammary epithelial cells to drive malignant progression.
- Created in vitro cell culture model systems that will be utilized in future studies to uncover additional genes and proteins involved in spindle checkpoint dysfunction that contribute to malignant progression in breast cancer.
- Demonstrated novel interaction of MAD2 with the microtubule associated protein EB1 and analyzed EB1 for involvement in breast cancer progression.

Reportable Outcomes:

This work has been presented at meetings and lectures:

Myrie K.A., Pimentel H. and Petty E.M. Association of the Spindle Checkpoint protein MAD2 with CDC20 and EB1: Implications for Tumorigenesis. The 93nd Annual Meeting of the American Association for Cancer Research; April 6-10, 2002; San Francisco, California

Myrie K.A., Loeb A. M. Fraker E. M., Neeley C.K. and Petty E.M. Analyses of MAD2L1 Expression and Associated Spindle Checkpoint Dysfunction in Ovarian Cancer Cells. The 92nd Annual Meeting of the American Association for Cancer Research; March 24-28,2001; New Orleans, Louisiana.

"Mishaps in mitosis: Contributions to cancer progression and implications for cancer therapy," Internal Medicine Grand Rounds, Vanderbilt University School of Medicine, Nashville, TN. 5/10/2001.

"The role of cell cycle regulation and apoptosis in carcinogenesis," Berlex Oncology Foundation Genetics Workshop, Stevenson, Washington, 6/8/2002

This work has led to the publication or preparation of the following:

Petty, E.M., Myrie, K.A. MAD2L1. Atlas Genet Cytogenet Oncol Hematol. March 2001. URL:http://www.infobiogen.fr.services/chromcancer/Tumors/MAD2L1ID304.html

Myrie K.A., Loeb A. M. Fraker E. M., Neeley C.K., Petty E.M. (2002) Analysis of MAD2L1 Expression and Spindle Checkpoint Dysfunction in Ovarian Cancer Cells. In preparation.

Myrie K.A., Brenner A.S., Petty E.M. (2002) Characterization of MAD1 and MAD2 expression in breast cancer cells. In preparation

Conclusions:

Mutation rates in somatic cells suggest that it is improbable for an mammary epithelial cell to accumulate enough mutations to cause malignant transformation within a women's lifetime unless events rendering the cell more mutable occur to drive malignant progression. Mutations can accumulate more rapidly in cells with defective genome caretaker genes that normally help maintain a cell's genetic stability. Most late stage breast cancers demonstrate aneuploidy, presumably related to increased chromosomal instability which could result from mitotic spindle checkpoint dysfunction. Several spindle checkpoint related proteins, including BUB (budding uninhibited by benomyl) and MAD (mitotic arrest-deficient) family members, normally regulate proper chromosome segregation by monitoring proper chromosome attachment to mitotic spindle microtubules prior to cell cycle progression via CDC20-activated anaphase. Alterations of MAD and BUB genes have been associated with instability, MSC dysfunction, apoptosis, cellular proliferation, tumorigenesis, and human cancers. Some chemotherapeutic agents currently used to treat mammary cancer target the mitotic spindle microtubules. The efficacy of these agents may depend in part on the function of the mitotic spindle checkpoint. Our initial analysis of the mitotic spindle checkpoint and associated genes/proteins in mammary cancer cells suggests some MSC dysfunction in close to 50% of cancer cell lines; reduced *MAD2L1*, *MAD1L1*, and *CDC20* expression in >30% of primary mammary carcinomas; and loss of MAD2 expression in mammary cancer lines with evidence of marked MSC dysfunction. The functional roles of the mitotic spindle, MAD2 and MAD2-associated proteins, and other spindle checkpoint proteins in genomic instability, malignant progression, and chemotherapeutic responsiveness of mammary cancer cells have yet to be elucidated. We have developed in vitro and in vivo model systems to help us further evaluate *MAD2L1, MAD1L1*, *CDC20,* and *MAPRE1* in mammary cancer cells and correlate findings with observed chromosomal instability and/or aneuploidy. These resources should help us and others further characterize how mitotic spindle checkpoint gene/protein alterations are associated with clinical, histopathological, and genetic profiles. In addition, these model systems may demonstrate utility in the future analysis of chemotherapeutic agents that target the spindle microtubules in mitotic spindle checkpoint deficient mammary epithelial cells.

We believe that characterization of the mitotic spindle checkpoint and associated regulatory genes/proteins in mammary epithelial cells should provide important insights into molecular mechanisms contributing to their genomic instability and deepen our understanding about pathways of malignant progression in breast cancer cells. In addition, since some of the common agents currently used to treat mammary cancer are microtubule spindle poisons we will explore how their efficacy correlates with the checkpoint's ability to recognize and respond to abnormal chromosome/spindle microtubule attachments. Fully characterizing and understanding cell cycle checkpoints that regulate cell proliferation, including the mitotic spindle checkpoint, in mammary cancer should prove useful when developing molecularly targeted biological weapons for improved management of mammary cancer and in developing innovative strategies for the prevention of disease associated morbidity and mortality. Thus, we hope that our work done during the course of this Concept Award will lead to future studies that may have significant prognostic and/or therapeutic relevance for women with breast cancer.

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